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(71) Applicants: BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US). MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US). SHRINERS HOSPITALS FOR CHILDREN [US/US]; 2900 Rocky Point Drive, Tampa, FL 33607 (US).

(72) Inventors: ORGILL, Dennis, P.; 217 Clatin Street, Belmont, MA 02178 (US). BUTLER, Charles, E.; 519 Washington Street, No. 5, Brookline, MA 02146 (US). YANNAS, Ioannis, V.; 7 Marcellus Drive, Newton, MA 02159 (US). COMPTON, Carolyn, C.; 18 Cramond Road, Chestnut Hill, MA 02167 (US).

(74) Agents: CARROLL, Alice, O. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

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(57) Abstract

The present invention relates to a method of tissue regeneration of a wound or burn in a mammal. This method comprises covering the wound with a membrane comprising a biodegradable, fibrous lattice and an optional outer moisture barrier, wherein the lattice is seeded with cultured cells obtained from a culture grown to subconfluence. This allows infiltration of the grafted matrix by mesenchymal cells and blood vessels from healthy underlying tissue, forming a neodermis, and proliferation of the keratinocytes, forming a neoepidermis. The resulting graft has excellent take rates and has the appearance, growth, maturation and differentiation of normal tissue.

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CULTURED CELL-SEEDED FIBROUS LATTICE FOR TISSUE REPLACEMENT

Background of the Invention

Mammals suffer tissue loss from a variety of mechanisms including trauma, tumor removal, vascular disease, genetic defects and infections. Replacement of lost tissue or organs is often essential for either survival or function of the mammal.

Many mammalian tissues can be thought of as bi-layer constructs. The surface layer contacts the environment or one or more body fluids, and the stromal layer provides mechanical support and a vascular supply to the surface layer(s). These bilayer tissue types include skin, trachea, bronchi, vermillion, oral lining, nasal lining, intestines, biliary ducts, ureters, bladder and blood vessels. When replacing any of these tissues or structures, it is essential that both the stromal and surface layers be reconstituted.

A patient who has suffered extensive tissue loss or injury is immediately threatened by infection and by excessive loss of fluids. To meet both of these needs, a large wound must be closed promptly by some type of membrane. Ideally this membrane should have characteristics of the surface layer (epidermis) and the stromal layer (dermis). The most direct method of accomplishing this purpose is to remove the injured tissue and graft sections of skin to the wound, restoring the function of the skin as a barrier to the outside environment. Such skin grafts contain the epidermis, or surface layer, as well as the dermis, or stromal layer.

Skin can be removed or harvested from an animal of another species. This type of transplant is referred to as a xenograft. However, a xenograft suffers from the disad-

vantage that the transplanted skin ultimately undergoes immunologic rejection and can only serve to cover the wound for three to ten days. Consequently, a xenograft can only serve as a stopgap measure while the patient's skin slowly heals.

Human skin, obtained from cadavers or relatives of the patient, can also be transplanted and is referred to as an allograft or homograft. However, cadaveric skin is in short supply, and allografts are often, like xenografts, rejected. Although immunosuppressive drugs can increase the period of time which an allograft may cover a wound, they also leave the patient vulnerable to infection. Allografts also suffer from the disadvantage that they expose the patient to the risk of transmission of diseases such as hepatitis and AIDS.

The most desirable form of transplant is an autograft, in which skin from an undamaged area of the patient or identical twin is harvested and used to cover the wound. The risk of rejection and disease transmission is thereby eliminated, and the transplanted skin proliferates to form a new layer of dermis and epidermis. However, the harvesting operation is a painful, invasive process which causes scarring. In addition, a severely burned patient may suffer tissue loss or damage on nearly all of his or her body. This may severely limit the amount of healthy, intact tissue that is available for autografting. When this occurs, xenografts or homografts may be placed across the entire wound surface to control infection and dehydration; they are gradually replaced as autografts become available. Autografts may be harvested repeatedly from a donor site. However, each donor site must be allowed to heal before another autograft is removed from it; this requires a substantial delay, and prolongs the recovery of the patient. Furthermore, the quality of the tissue graft diminishes with each successive harvest.

Consequently, attempts have been made to manufacture artificial skin from both biologic and synthetic materials

with variable results. An acceptable skin substitute should provide both the components and functional results of normal skin, provide for early wound closure and minimize long term scarring and contraction. In addition, an effective skin replacement should be free from risk of disease transmission and immunologic rejection.

One promising technology for manufacturing and applying artificial skin is referred to as cultured epithelial autograft (hereinafter referred to as "CEA") (Green et al., Proc. Natl. Acad. Sci. USA 76:5665-5668 (1979); O'Connor et al., Lancet 1:75-78 (1981)). In this method, skin biopsies are harvested from an uninjured site on the patient's body. The epithelial cells from this graft are grown to confluence in culture to form epithelial sheets that are applied directly to the wound bed, basal side down.

The CEA method suffers from the limitations of requiring several weeks of preparation time and providing only epidermal coverage to the wound bed. There is no dermis or basement membrane present at the time of application. Thus, there is nothing to anchor the new epidermis, or neoepidermis, to the underlying tissue. This results in poor take rates for CEA sheets applied directly onto wounds. Clinically, this is seen as shearing and blistering of the transplanted CEA. Consequently, efforts have been made to use dermal substrates, such as cadaveric skin, to improve take rates (Kangesu et al., Br. J. Plas. Surg. 46:393-400 (1993); Kangesu et al., Br. J. Plas. Surg. 46:401-409 (1993); Cuono et al., Plast. Reconstr. Surg. 80:626-637 (1987)). However, allograft rejection, the risk of disease transmission and limited availability of cadaveric skin are serious limitations on the usefulness of this technique. Furthermore, the combined use of CEA and cadaver allografts require at least two grafting procedures to produce a dual layer skin substitute.

One of the most promising skin substitutes is a synthetic bi-layer membrane (hereinafter collectively referred to as "CG bi-layer") (see U.S. Patent Nos. 4,060,081 and

4,280,954 to Yannas et al.). This membrane comprises a bottom layer (hereinafter referred to as "CG matrix" or "CG lattice") which is a highly porous lattice made of collagen and glycosaminoglycan. The top or outer layer is a membrane semipermeable to moisture and impermeable to infectious agents such as bacteria. The CG lattice serves as a supporting or scaffolding structure into which blood vessels and mesenchymal cells migrate from below the wound, a process referred to as "infiltration". Infiltration is responsible for creating a new dermis, referred to as the "neodermis", which replaces the CG matrix as it biodegrades. Epithelial cells from undamaged skin surrounding the edges of the wound migrate into CG matrix to create a new epidermis, referred as the "neoepidermis". Because burns and other skin wounds tend to be shallow, mesenchymal cells need not migrate very far to create a neodermis. However, burns often cover large areas of a patient's body surface. Consequently, epithelial cells often must migrate great distances to adequately close a wound. As a result, prior skin replacement techniques utilizing a CG matrix provide only a dermal layer and require thin skin grafts to close the wound. To provide an epidermis, a thin, split-thickness autograft can be applied after the matrix is vascularized; however, complete wound closure remains dependent on healing time and size of donor sites and requires multiple grafting procedures (Burke et al., Ann. Surg. 194:413-428 (1981); Heimbach et al., Ann. Surg. 208:313-320 (1988)).

Alternatively, CEA can be grafted to the vascularized CG matrix to provide an epidermis, resulting in a dual-layer skin substitute (see U.S. Patent No. 5,489,304 to Orgill et al.) However, as with other techniques, at least two grafting processes are required to produce satisfactory results. Additionally, the technique suffers from the limitation associated with CEA use, including lengthy preparation time, susceptibility to infection, variable

graft survival and potential epidermal fragility and blistering.

Various composite grafts with both a dermal and epidermal layer have been studied. The dermal components have been prepared by incorporating dermal fibroblasts onto various substrates including laminated matrix, CG matrix, polyglycolic acid or polygalactin mesh or collagen gel (see U.S. Patent Nos. 4,418,691 (Yannas et al.); 4,458,678 and 4,505,266 (Yannas and Burke); and 5,273,900 (Boyce et al.)). Keratinocytes are then placed on the surface and cultured, *in vitro*, until a confluent epidermis, several layers thick, is formed. In some instances, an effort has been made to select for basal or stem cell populations, e.g., by density centrifugation or antibodies; however, these methods do not efficiently isolate basal cells and do not provide a significant expansion factor for cell proliferation. The limitations of these composite grafts have been the lengthy time for production and poor graft survival as a result of growth of the cells to confluence. Consequently, a need exists for new skin replacement compositions and techniques.

Summary of the Invention

The present invention pertains to a method for regenerating multi-layered, often specialized, tissue from a biodegradable, fibrous lattice seeded with isolated site-specific, autologous donor cells. The donor cells are obtained from cultured cells grown only to subconfluence but not to confluence. The fibrous lattice may optionally comprise an outer moisture barrier.

It is imperative to the present invention that the cell cultures from which the cells, e.g., keratinocytes or fibroblasts, are obtained are grown only to subconfluence and not to confluence. This provides for a higher fraction of basal cells or stem cells available for seeding compared to that of uncultured keratinocytes or cultured keratinocytes grown to confluence. When keratinocyte cultures are

grown to subconfluence there is selection for a stem cell population which is undifferentiated and capable of cellular division. As cultures become confluent, cells experience contact inhibition, and many undergo apoptosis or programmed cell death, reducing cellular proliferation. The stem cells begin to differentiate, thus reducing their ability to divide. Seeding the fibrous lattice with cells, e.g., keratinocytes, from subconfluent cell culture not only greatly enhances the cellular expansion capability but also greatly increases the fraction of seeded cells which are capable of cellular proliferation and colony formation. The higher ratio of basal cells in cells from subconfluent cell cultures allows a lower seeding density of cells since a larger proportion are undifferentiated and capable of proliferation.

Thus, several advantages can be realized from the present invention. First, the fibrous lattice or matrix can be seeded with a lower density of cells when those cells are derived from a subconfluent culture as compared with cells from a confluent culture. Additionally, utilizing cells from subconfluent cell culture greatly enhances the cellular expansion capability of the cells, allowing fewer cells to cover greater wound surface area more quickly. This is particularly advantageous for tissue regeneration in burn victims, whose wounds can be extensive and who may have few uninjured sites from which to obtain the initial cells.

In particular, the invention relates to a method of replacing and regenerating tissue at a burn or wound on a mammal, e.g., a human or animal. A large surface area expansion factor is achieved as a result of cellular expansion in cell culture, and selection of a proliferating cell population, i.e., a cell population grown to subconfluence; this result is not achievable utilizing presently known methods. A confluent, differentiated tissue layer incorporated onto a vascularized mesenchymal support structure is formed two weeks after the cell-seeded fibrous lattice is

grafted. The regenerated tissue has a structure and function similar to that of the lost native tissue. Furthermore, tissue, particularly epithelial or dermal tissue, lost from any cause can be replaced with this method using minimal autologous donor tissue. The present invention also relates to synthetic tissue comprising a biodegradable, fibrous lattice or matrix seeded with cultured cells obtained from a culture grown to subconfluence and optionally comprising an outer moisture barrier.

Many mammalian tissues can be thought of as bi-layer constructs. The surface layer contacts the environment or one or more body fluids, and the stromal layer provides mechanical support and a vascular supply to the surface layer(s). These bilayer tissue types include skin, trachea, bronchi, vermillion, oral lining, nasal lining, intestines, biliary ducts, ureters, bladder and blood vessels. When replacing any of these tissues or structures, it is essential that both the stromal and surface layers be reconstituted. As used herein, skin will be described in detail as an example of a bilayer tissue replacement. However, this invention is not construed to be limited to skin as the appropriate tissue; the invention is intended to encompass any and all of the bi-layer tissue constructions known in the art.

As used herein, terms are intended to have their art-recognized meaning unless otherwise defined. As used herein, "confluence" is intended to mean a merged or non-discrete cell layer. As used herein, "subconfluence" is intended to mean cells or a cell layer which has not grown to a point of confluence and contains separate and distinct cells or cell aggregates. Also, as used herein, "site-specific" cells are intended to mean cells that produce a site-specific phenotype, e.g., cells that produce specialized epithelial structures such as lips, bowel mucosa or bladder tissue.

The method of the present invention comprises the steps of applying a biodegradable, fibrous lattice or

matrix, seeded with cultured cells isolated from a culture grown to subconfluence, and comprising an optional semipermeable outer moisture barrier (e.g., a silicone layer) to a wound so that the semipermeable moisture barrier is exposed to the air. In particular embodiments, the matrix is seeded with keratinocytes or fibroblasts grown to subconfluence in culture.

The seeding is generally carried out before the matrix is applied to the wound but can also be performed after the matrix has been grafted or affixed to the wound site. Blood vessels and mesenchymal cells from the site surrounding the afflicted area are allowed to infiltrate the lattice or matrix from tissue beneath the matrix, forming a neodermis, and the cultured keratinocytes proliferate to form a neoepidermis. Over time a neodermis and neoepidermis are formed at the burn or wound site, resulting in tissue having the appearance, differentiation and growth of normal tissue, e.g., skin. This process allows for the simultaneous formation of a specialized epithelium overlying a vascularized mesenchymal tissue layer, and this result is achieved utilizing only one grafting procedure.

This method can be used to repair or replace many different types of specialized epithelium, including, but not limited to, uroepithelium (bladder, urethra, ureter), gastrointestinal mucosa (oropharynx, esophagus, stomach, intestine), respiratory epithelium (trachea, bronchus) and vasculature (artery, vein, lymphatics). Accordingly, the type of cell which is seeded onto the matrix will vary according to the tissue to be replaced and can be readily ascertained by the skilled artisan.

For example, abnormal tissue can be intentionally (e.g., surgically) removed from an individual and new tissue can be produced in its place using this method. Alternatively, the method of the present invention can be used to produce new tissue in place of tissue which has been lost due to accident or disease. In addition, other types of cells can be co-seeded into the matrix to allow

the mesenchymal tissue layer to have specialized function or functions. For example, chondrocytes could be used for cartilage production or endocrine cells could be seeded to provide hormone production.

The present invention has many advantages. It allows immediate wound coverage, requires only one grafting process, and produces a result similar to native tissue. The fibrous lattice or matrix is completely synthetic and biodegradable over time. The present method can utilize autologous donor cells, and there is therefore no risk of disease transmission from donor to patient.

Detailed Description of the Invention

Two important components of the skin are the epidermis and dermis. The epidermis is the outer layer of skin. It consists of cells at various stages of differentiation and maturity. Basal cells are located at the lowest level (adjacent to the dermis) and are the least differentiated. The dermis is located below the epidermis and comprises mesenchymal cells and blood vessels. The junction between the dermis and epidermis is referred to as the basement membrane and is responsible for one of the most important functional results of normal skin, namely the tight adhesion of the dermis to the epidermis. This tight adhesion adds strength and durability to the skin and prevents "shearing" of the epidermis. "Shearing" is the rubbing off of the epidermis when lateral forces are applied to the skin, and can result in blistering and skin fragility.

In normal epidermis, keratinocytes undergo a sequence of differentiation from the undifferentiated basal cell near the dermal-epidermal junction to the anucleate corneal cell at the surface. Under normal conditions, only basal cells are capable of cellular division. To achieve a constant epidermal thickness, the rate of basal cell replication is approximately equal to the rate of cells being shed at the stratum corneum. Generally, only a small fraction of viable keratinocytes obtained from disaggre-

gating epidermis are basal cells which are able to proliferate into keratinocyte colonies. Therefore, it is the seeding density of the basal cell population and not the total number of viable keratinocytes, which is important in the formation of a confluent epidermis.

As described herein, a superior cultured cell-seeded matrix for use in mammalian full-thickness wounds has been developed and tested on a porcine model. The porcine model closely approximates human full-thickness wounds and allows multiple wounds to be directly compared on the same animal. As also described herein, a method of regenerating epithelial tissue has been developed which optimizes epidermal proliferation, minimizes the time required for epidermal confluence and maximizes epidermal surface area expansion. The present invention provides a potentially unlimited epidermal expansion capability by applying the technique of cell culturing to the seeding of porous matrices. Using cultured cells, an essentially unlimited area of seeded-matrix grafts can be produced from a single small biopsy site, and such grafts provide permanent closure of full-thickness wounds in a single grafting procedure by simultaneous formation of a neodermis and neoepidermis.

Furthermore, cultured cell-seeded matrices can be grafted immediately after cell seeding without additional in vitro production time. Large surface area tissue defects can be completely closed with seeded grafts. Alternatively, uncultured keratinocyte seeded grafts can be prepared and grafted immediately after skin donor harvest, if desired, and cultured keratinocyte-seeded grafts used as early as one week later. Moreover, keratinocyte cell culture provides an ever-expanding population of autologous keratinocytes for seeded graft production and for repeated grafting procedures without additional donor site harvests.

Accordingly, the present invention pertains to a method of epithelial tissue regeneration, particularly for burns or wounds on a mammal, including humans and domestic and veterinary animals. This invention overcomes many of

the shortcomings presented by methods presently used to regenerate tissue to cover burns or wounds. According to the method of the present invention, a highly porous, fibrous lattice which is seeded with cells grown to sub-confluence in culture and which is optionally covered with an outer membrane that is a moisture barrier, is applied to a wound. The wound is covered with the lattice applied directly to the wound and with the moisture barrier, if present, exposed to the air.

The lattice serves as a temporary substitute for the dermis and can be any structure that has the following characteristics. The composition and structure of the lattice must be such that is does not provoke a substantial immune response from the graft recipient. The lattice must be sufficiently porous to permit blood vessels and mesenchymal cells from healthy tissue below the wound to migrate into the lattice. As discussed herein, this migration is referred to as "infiltration" and is responsible for the generation of the neodermis. The lattice can be either synthetic or biological in origin. For example, cadaver dermis can be used as a "natural" or biological fibrous matrix of the present invention.

To facilitate the formation of the neodermis, synthetic lattices should be biodegradable. This biodegradation must not proceed so rapidly that the lattice disappears before sufficient healing occurs, i.e. before sufficient neodermis forms. Lattices that degrade too slowly impede cell migration and cause the formation of a fibrotic layer of cells surrounding the lattice. A lattice which biodegrades after about thirty days is preferable.

The optional moisture barrier is any composition which can serve as an outer surface to the lattice and must be capable of being manually removed at will from the lattice. Compositions suitable for use as a moisture barrier must also have the property of being semipermeable to the passage through the wound of fluids from inside the body and impermeable to microorganisms such as bacteria and viruses

from outside the body. The moisture barrier also imparts several desirable physical properties to the lattice such as tensile strength and suturability. The moisture barrier layer may not be necessary for internal uses or applications in which the tissue or organ is not exposed to air, and thus it is optional in such applications. Silicone elastomers are suitable for use in the moisture barrier of the present invention.

For example, one embodiment of the present invention employs a highly porous fibrous lattice comprised of collagen and glycosaminoglycan (referred to hereinafter as "GAG"), i.e. a collagen glycosaminoglycan matrix (referred to hereinafter as "CG matrix") with a silicone elastomer as the outer membrane. A CG matrix with an outer silicone surface is prepared according to methods known to those skilled in the art (see U.S. Patent Nos. 4,060,081 and 4,280,954 (Yannas et al.) and 4,505,266 (Yannas and Burke), the teachings of which are incorporated herein in their entirety). Various forms of GAG which may be suitable for use in this material include chondroitin 6-sulfate, chondroitin 4-sulfate, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chitin and chitosan.

It is possible to control several parameters of the CG matrix (primarily crosslinking density, porosity and GAG content) to control the rate of biodegradation of the lattice. Specific conditions for forming a CG matrix suitable for use in the present invention are given in the Exemplification. However, the skilled artisan will know of other conditions for forming CG matrices with variations of the above-mentioned parameters which are similarly suitable for use in the present invention. In addition, certain applications of tissue regeneration may require matrices which degrade more slowly or more quickly. The skilled artisan will be able to recognize applications where it is desirable to vary the properties of the CG matrix, and will be able to vary the parameters accordingly; the present

invention is intended to encompass such variations in the CG matrix.

Although the research that led to this invention involved CG matrices and silicone outer membranes, the tissue regeneration method of this invention is not limited to CG matrices and silicone outer membranes. Other fibrous proteins, polymeric molecules, biological compositions or sintered ceramics having appropriate properties can be used in the present invention, and such lattices and materials are within the scope of this invention. In addition, for internal tissues it is possible to utilize the seeded fibrous lattice without the outer moisture barrier.

The matrices are then seeded with appropriate cells. Generally, appropriate cells will be keratinocytes or fibroblasts but may also include other cell types depending upon the tissue to be replaced. For example, site-specific cells such as those found in the palm, sole, face, vermillion, mucosa, conjunctiva, small intestine, bladder, endothelium, cartilage and bone can be seeded onto the matrix, either alone or in combination, depending upon the desired characteristics of the final product. "Cultured cell-seeded matrices" refer to matrices into which cells, which have been harvested from a site on the patient's body surface and grown in culture to subconfluence, have been introduced. Procedures for seeding matrices are described in U.S. Patent No. 4,060,081, the teachings of which are incorporated herein by reference in its entirety. Matrices which have been seeded are referred to as "cellular" while unseeded matrices are referred to as "acellular". Seeding will generally be carried out prior to the time at which the matrix is grafted to the wound, although the seeding process can be performed after grafting.

Cultured cell-seeded matrices may be autologous, i.e., matrices seeded with cells obtained from the human or animal having the burn or wound, or they may be heterologous, i.e. seeded with cells obtained from a donor. In addition, cells being used to seed a matrix may undergo

genetic manipulation or modification in order to prevent rejection or to change the cell's phenotype in some beneficial manner. Genetic manipulation or modification includes, but is not limited to, introducing genetic matter into the cells so that the protein gene product or products are expressed in sufficient quantities to cause the desired change in phenotype, for example as described by Lyerly and DiMaio (Arch. Surg. 128:1197-1206 (1993)), the teachings of which are incorporated herein by reference. An example of suitable genetic matter includes the gene encoding for a growth factor along with the requisite control elements, as described in Morgan et al. (Science 237:1476 (1987)), the teachings of which are incorporated herein by reference. Other examples of suitable genetic material include, but are not limited to, the E1A oncogene (Barrandon et al., Proc. Natl. Acad. Sci. USA 85:4102 (1989)) and the neo gene (Flowers et al., Proc. Natl. Acad. Sci. USA 87:2349 (1990)), the β-galactosidase gene (Vogt et al., Proc. Natl. Acad. Sci. USA 91:9307-9311 (1994)) and the hGH gene (Vogt et al. (1994)).

It is imperative to the present invention that the cell cultures from which the cells, e.g., keratinocytes or fibroblasts, are obtained are grown only to subconfluence and not to confluence. This provides for a higher fraction of basal cells or stem cells available for seeding compared to that of uncultured keratinocytes or cultured keratinocytes grown to confluence. When keratinocyte cultures are grown to subconfluence there is selection for a stem cell population which is undifferentiated and capable of cellular division. As cultures become confluent, cells experience contact inhibition and many undergo apoptosis or programmed cell death, reducing cellular proliferation. The stem cells begin to differentiate, thus reducing their ability to divide. Seeding cells, e.g., keratinocytes, obtained from subconfluent cell culture not only greatly enhances the cellular expansion capability but also greatly increases the fraction of seeded cells which are capable of

cellular proliferation and colony formation. The higher ratio of basal cells in cells from subconfluent cell cultures allows a lower seeding density of cells since a larger proportion are undifferentiated and capable of proliferation.

The cell culture can be adjusted and controlled to inhibit cellular differentiation. One useful method of controlling cellular differentiation in keratinocytes is limitation of calcium concentration; however, other methods will be readily apparent to the skilled artisan and these methods are encompassed by the present invention. For example, prior to confluence, cells can be released from a cell culture plate and replated in a more dilute manner in multiple flasks; this process can be repeated multiple times to control cellular differentiation.

Keratinocytes can be harvested and stored for short periods of time (sufficient for subconfluence but not confluence) in active cell culture or for prolonged periods by freezing the cells. This allows keratinocytes to be expanded in cell culture and stored prior to the time when grafting is needed. If cells have been previously harvested and expanded, then seeded grafts can be prepared and applied immediately, without additional cell harvesting or cell culture time. This technique is particularly useful when the need for grafting can be anticipated or planned in advance such as for elective reconstructive surgery or when future grafting for burn patients may be necessary.

Once the CG matrix has been prepared (and, usually, seeded), the wound is readied for application of the matrix. Areas of tissue that have been destroyed or damaged are surgically removed to prevent them from interfering with the healing process. The entire area of dead and damaged tissue is excised, so that intact epithelial cells are present at the perimeter of the wound. The CG matrix, with the optional silicone side, if present, away from the wound, is draped across the wound to avoid the entrapment of air pockets between the wound and the matrix. The

lattice is sutured or stapled to the wound using conventional techniques and then covered with a bandage.

For example, a burn patient may lack the vermillion of the lower lip (the pink area of the lip). This defect can result in severe deformity and contraction of the lip. A small biopsy from the upper lip could be taken and disaggregated in vitro. In culture, the epithelial vermillion cells can be expanded. When sufficient cells are present, these cells can be seeded into an appropriate porous lattice which is sutured into the lip defect. In situ, the cells proliferate to close the wound.

After application of the fibrous lattice, blood vessels and mesenchymal cells from underlying healthy tissue begin, as described herein above, the process of infiltration of the grafted matrix. "Infiltration", as defined herein, further refers to a sufficient period of time for this migration of mesenchymal cells and blood vessels. A preferred period of time is about ten days, but periods as short as about two to three days are also appropriate.

It is also contemplated by the present invention that CEA or other confluent sheets of epithelium can be used in conjunction with the present invention to form an artificial skin bi-layer if desired. See Roberto George Casper, "Cultured Keratinocyte Grafting: Implications for Wound Healing," Profschrift, Denhang, Netherlands, 1993, and U.S. Patent No. 5,489,304 (Orgill et al.) the teachings of which are hereby incorporated by reference in their entirety.

The formation and progression of the replacement tissue is indicated by visually observing epidermis on the wound surface which persists for several days post-grafting. The extent to which a graft progresses can be more precisely determined by the amounts of wound surface area which is epithelized, i.e., how much of the wound surface area is covered by neoepidermis. This can be determined by histological means and is described more fully in the Exemplification. A graft which "takes", i.e., survives and flourishes, is typically characterized by the presence of

epithelial cells covering the neodermis. Eventually there is a formation of a basement membrane at the junction of the neoepidermis and dermis, including components such as anchoring fibrils. This results in a tight union between the neoepidermis and neodermis.

Once the neodermis and neoepidermis have progressed sufficiently to the point where the replacement skin can function to protect the body against infection or infiltration from micro-organism and moderate fluid passage, the optional moisture barrier (e.g. silicone outer layer) is manually removed from the matrix. The wound can then be covered with a dry sterile gauze which is changed periodically.

The present invention has application to massively burned patients as well as to patients undergoing reconstructive surgery, chronic skin diseases and chronic wounds. The present invention will also be useful in the replacement of other specialized epithelial tissues in a variety of organ systems, including, but not limited to, uroepithelial, gastrointestinal, respiratory and vascular. Tissue loss from malignancy, congenital or acquired disease and surgical removal can be replaced with tissue composed of the same specialized native cells.

The method of the present invention is particularly useful to provide skin coverage for patients with limited skin graft donor sites, such as massive burn victims. The reconstructive surgeon would be able to replace large skin defects without the need for large skin grafts. Skin loss from acute and chronic skin diseases could be replaced with cultured cell-seeded CG grafts.

Specialized epithelial tissue such as bladder, ureter, oral mucosa, esophagus, trachea, blood vessel and intestine often requires replacement or reconstruction after surgical excision. Currently, this tissue can only be replaced with prosthetic material or a section of autologous tissue from another location with similar functional characteristics, often from a different organ system. The use of prosthetic

material is limited by its non-viability, lack of specialized function, immunologic reaction or rejection and increased risk of infection. Autologous tissue from a separate location is often used to replace tissue defects. For example, intestine can be used for esophageal replacement and bladder reconstruction, and urinary conduit can be used for ureter loss or bile duct replacement. Also, donor veins are used to replace arteries. Using autologous tissue for replacement requires a surgical procedure and tissue loss from an uninjured organ. In addition, the donor tissue often does not have the identical structural or function characteristics of the native tissue and suffers from lack of specific anatomic and physiologic function.

Specific cultured cell-seeded matrices (i.e., matrices seeded with specialized, site-specific epithelial cells as described herein) can be used by the oncologic, trauma or reconstructive surgeon to replace tissue defects with a tissue composed of organ-specific cells identical to the native tissue, without the need to violate uninjured organs for donor tissue. Such tissue can be replaced after surgical resection for malignancy, disease or trauma. This method allows for replacement of various commonly lost tissues such as oropharyngeal, nasal and bronchial mucosa, lip vermillion, blood vessels, trachea, esophagus, stomach, small and large bowel, biliary ducts, ureter, bladder, urethra, periosteum, synovium, areolar tissue and vaginal mucosa. The lattice or matrix is flexible enough to be molded into the appropriate shape or form and then secured to adjacent or contiguous uninjured tissue while tissue regeneration progresses.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein in their entirety by reference.

Exemplification

Example 1: Preparation of CG Matrix

Bovine hide collagen, 0.5% by weight was dispersed in 0.05 M acetic acid and co-precipitated with chondroitin-6-sulfate. The co-precipitate was concentrated by centrifugation, and excess acetic acid was decanted. The concentrated co-precipitate was poured into flat, stainless steel freezing pans to a volume of 0.3 ml per square centimeter and placed on the cooled (-30°C) shelf of a freeze-drier. The frozen aqueous component of the co-precipitate was sublimated under vacuum to produce a highly porous matrix 2-3 mm thick. The constituent molecules of the matrix were cross-linked using a 24 hour dehydrothermal treatment at 105°C and 30 milliTorr. The sterile material was coated with a thin (approximately 0.3 mm thick) layer of silicone, which was cured in 0.05 M acetic acid at room temperature for 24 hours. The matrix was further cross-linked by a 24 hour treatment with a 0.25% (by volume) glutaraldehyde solution in 0.05 M acetic acid. The ECM analog was then exhaustively dialyzed in sterile, de-ionized water and stored in sterile 70% isopropanol until used. Before grafting, the matrix was washed and bathed in phosphate buffered saline (PBS) to remove the alcohol.

Example 2: Keratinocyte Processing

Split-thickness skin grafts (0.25 mm) were harvested, and rinsed in phosphate buffered saline with antibiotics, and the epidermis was separated from dermis with 0.25% dispase at 37°C for 2 hours. The epidermis was shredded with tissue forceps and keratinocytes were disassociated with Trypsin (0.1%) and EDTA (0.02%) at 37°C for 30 min. Weymouth's medium supplemented with 20% fetal calf serum and growth substances was added to neutralize the Trypsin, then the solution was filtered through a 100 μm cell filter, centrifuged at 1200 RPM for 5 minutes, and re-suspended in media. Viable cells were counted using Trypan Blue

exclusion. The resultant keratinocyte solution can be used for both initiation of keratinocyte cell cultures and for directly seeding matrix grafts (uncultured keratinocyte-seeded matrix grafts).

Example 3: Preparation of Uncultured Keratinocyte-Seeded Grafts

The keratinocyte solutions described in Example 2 were seeded into the porous matrix side of the CG matrix graft using a seeding density of 100,000 uncultured keratinocytes/cm^2 of CG matrix. Keratinocytes were centrifuged at 400 RPM for 15 minutes through the CG matrix to the matrix-silicone interface using specially designed sterile trays to maintain the gravitational vector perpendicular to the graft surface. Seeded grafts were secured to dorsal full-thickness wounds with surgical clips and dressed with a petroleum impregnated gauze and elastic bandage.

Example 4: Preparation of Cultured Keratinocyte-Seeded Grafts

Keratinocytes suspended in Weymouth's media with 20% fetal bovine serum and growth substances as described in Example 2 were plated at a density ranging from 1 x 10^4 to 2 x 10^7 cells per 75 cm^2 tissue culture flask. The flasks were placed in cell culture at 37°C, 95% humidity and 5% carbon dioxide, and the medium was changed every 2 days. When the cells were approximately 80% confluent (approximately 10-14 days) they were released from the flask and disaggregated with Trypsin (0.1%) and EDTA (0.02%) at 37°C for 20 minutes. The cell solution was filtered, centrifuged at 1200 RPM for 5 minutes, re-suspended in fresh media, and viable cells counted. This solution was used for seeding CG matrix grafts (cultured keratinocyte-seeded grafts) and additional passaging of keratinocyte cultures (subculture). Seeded grafts were secured to dorsal full-thickness wounds with surgical clips and dressed with a petroleum impregnated gauze and elastic bandage.

Example 5: Comparison of Cultured vs. Uncultured Keratinocyte-Seeded Grafts

Fifteen days prior to the grafting, a split-thickness skin biopsy was taken from the scapular region of the pig and keratinocyte cell cultures initiated as previously described. On the day of grafting a second split-thickness skin biopsy was harvested and processed as described to prepare uncultured keratinocyte seeded CG matrix grafts. Sixteen full-thickness, 3 x 3 cm dorsal paraspinal wounds were excised to subcutaneous fat with a surgical blade. Keratinocyte seeded CG matrix grafts, 3 x 3 cm, were placed on the wounds and secured with stainless steel surgical clips. Eight grafts were seeded with uncultured keratinocytes and eight with (first-pass) cultured keratinocytes prepared as described above. A seeding density of 100,000 cells/cm^2 was used for all grafts. Gross photographs and biopsies were taken at days 4, 7, 14, 20 and 26.

All grafts remained completely adherent to the wound bed and there was no gross evidence of sub-graft fluid collection or graft infection. The silicone layer was easily removed from all grafts at day 14. By day 14, a confluent, translucent epidermis with numerous keratin surface granules was grossly observed on all graft sites. The underlying neodermis was pink and was well vascularized. Keratin surface granules were observed on both cultured and uncultured keratinocyte seeded grafts only at days 14 and 20. The cultured keratinocyte seeded grafts were noted to have a thicker epidermis and greater number of keratin surface granules at days 14 and 20. At day 20 the cultured keratinocyte seeded grafts had a thicker and more opaque epidermis.

H+E sections from day 14 were examined histologically and compared. Statistical analysis was performed using the unpaired students t-test (n=5). The average epidermal thickness of the cultured grafts was 67% thicker than that of the uncultured grafts (1.81μm ± 0.19μm vs. 1.09μm ± 0.18μm, p=0.003). The number of keratinocyte cysts/mm

cross-section in the neodermis was greater (p=0.005) in cultured grafts (2.42 ± 0.67) than uncultured grafts (0.86 ± 0.63). The epidermis at 14 days was 96.4% ± 3.4% confluent in the cultured grafts and 49.8% ± 17.3% confluent in uncultured grafts (p=0.0004).

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

The invention claimed is:

1. A membrane comprising a fibrous lattice and an optional outer moisture barrier, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence.

2. Synthetic skin comprising a membrane comprising a fibrous lattice and an optional outer moisture barrier, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence.

3. A membrane comprising a fibrous lattice and an optional outer moisture barrier, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence, for use in regenerating tissue at a wound or burn site.

4. Synthetic skin comprising a membrane comprising a fibrous lattice and an optional outer moisture barrier, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence, for use in regenerating tissue at a wound or burn site.

5. The membrane or synthetic skin according to any one of Claims 1 to 4, wherein the fibrous lattice comprises a collagen-glycosaminoglycan matrix.

6. The membrane or synthetic skin according to any one of Claims 1 to 5, wherein the optional outer moisture barrier is removable and comprises a silicone elastomer.

7. The membrane or synthetic skin according to any one of Claims 1 to 6, wherein the cultured cells are keratinocytes and/or fibroblasts.

8. The membrane or synthetic skin according to any one of Claims 1 to 7, wherein the fibrous lattice is synthetic and biodegradable.

9. The membrane or synthetic skin according to any one of Claims 1 to 8, wherein the fibrous lattice is biological.

10. The membrane or synthetic skin according to any one of Claims 1 to 9, wherein the seeded cells have been genetically modified.

11. Use of a membrane or synthetic skin for regenerating tissue at a burn or wound site in a mammal, wherein the membrane or synthetic skin comprises a fibrous lattice and an optional outer moisture barrier, and wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence.

12. The use according to Claim 11, wherein the fibrous lattice comprises a collagen-glycosaminoglycan matrix.

13. The use according to Claim 11 or Claim 12, wherein the optional outer moisture barrier is a silicone elastomer.

14. The use according to any one of Claims 11 to 13, wherein the cultured cells are keratinocytes and/or fibroblasts.

15. The use according to any one of Claims 11 to 14, wherein the fibrous lattice is seeded with cultured

cells obtained from a culture grown to subconfluence after the membrane is applied to the wound or burn site.

16. The use according to any one of Claims 11 to 15, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence before the membrane is applied to the wound or burn site.

17. The use according to any one of Claims 11 to 16, wherein the optional outer moisture barrier is removable.

18. A method for regenerating tissue at a burn or wound site in a mammal, comprising applying to a wound or burn site a membrane comprising a fibrous lattice and an optional outer moisture barrier, wherein the moisture barrier, if present, is exposed to the air and wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence, whereby over time a neodermis and neoepidermis are formed at the burn or wound site, resulting in tissue that has appearance, growth and differentiation similar to normal tissue.

19. A method for regenerating tissue at a burn or wound site in a mammal, comprising applying to a wound or burn site a fibrous lattice seeded with cultured cells obtained from a culture grown to subconfluence, whereby over time a neodermis and neoepidermis are formed at the burn or wound site, resulting in tissue that has appearance, growth and differentiation similar to normal tissue.

20. The method according to Claim 18 or Claim 19, wherein the fibrous lattice comprises a collagen-glycosaminoglycan matrix.

21. The method according to any one of Claims 18 to 20, wherein the optional outer moisture barrier is a silicone elastomer.

22. The method according to any one of Claims 18 to 21, wherein the cultured cells are keratinocytes and/or fibroblasts.

23. The method according to any one of Claims 18 to 22, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence after the membrane is applied to the wound or burn site.

24. The method according to any one of Claims 18 to 23, wherein the fibrous lattice is seeded with cultured cells obtained from a culture grown to subconfluence before the membrane is applied to the wound or burn site.

25. The method according to any one of Claims 18 to 24, wherein the optional outer moisture barrier is removable.

26. The method according to any one of Claims 18 to 25, wherein the fibrous lattice is synthetic and biodegradable.

27. The method according to any one of Claims 18 to 26, wherein the fibrous lattice is biological.

28. A method according to any one of Claims 18 to 27, wherein the seeded cells have been genetically modified.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/15994

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 C12N5/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S T BOYCE AND J F HANSBROUGH: "BIOLOGIC ATTACHMENT, GROWTH AND DIFFERENTIATION OF CULTURED HUMAN EPIDERMAL KERATINOCYTES ON A GRAFTABLE COLLAGEN AND CHONDROITIN-6-SULFATE SUBSTRATE" SURGERY, vol. 103, no. 4, April 1988, pages 421-431, XP002050503 see the whole document ---	1-28
X	EP 0 681 846 A (SMITH & NEPHEW) 15 November 1995 see page 5, line 56 - line 58; claims; examples --- -/--	1-4,7, 11, 14-16, 18,19, 22-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1997	15.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 963 489 A (NAUGHTON GAIL K ET AL) 16 October 1990 see claims; example	1-4,7,8, 14-16, 18,19, 22-24
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International Application No
PCT/US 97/15994

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 489 304 A (ORGILL DENNIS P ET AL) 6 February 1996 cited in the application	

INTERNATIONAL SEARCH REPORT

I· -national application No.
PCT/US 97/ 15994

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 18-28
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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Information on patent family members

Inter nal Application No
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